Biologicals 42 (2014) 74–78

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Quantitative detection of residual porcine host cell DNA by real-time PCR

Jen-Ting Chang, Yu-Chen Chen, Yu-Chi Chou, Shih-Rong Wang*

Animal Technology Institute, Chunan, Miaoli, Taiwan

ARTICLE INFO

Article history:
Received 29 June 2012
Received in revised form
12 March 2013
Accepted 24 October 2013

Keywords:
Residual DNA
Q-PCR
transgenic pig
PERV

ABSTRACT

All biological products are derived from complex living systems and are often mixed with large numbers of impurities. For reasons of safety, residual host-cell DNA must be eliminated during processing. To assay host-cell DNA content in biopharmaceutical products derived from porcine sources, this study applies the quantitative real-time polymerase chain reaction (Q-PCR) method. The optimized assay in this study is based on the *pol* region of the porcine endogenous retrovirus (PERV). Assay validation results demonstrate that the proposed assay has appropriate accuracy, preciseness, reproducibility, and sensitivity. Primer and probe specificity are evaluated in real-time Q-PCR reactions using genomic DNA from rabbit, mouse, cat, hamster, monkey, human cell, yeast, and *Escherichia coli* as templates. The sensitivity of real-time Q-PCR is determined using genomic DNA from the porcine kidney cell line. The reliable detection range is within 0.5–10^5 pg/reaction. The limit of quantitation is 500 fg. The sensitivity of the assay meets the authority criterion. Moreover, the assay is applied to determine the level of host-cell DNA in recombinant human coagulation factor IX (rhFIX) from transgenic pigs. The real-time Q-PCR assay is thus a promising new tool for quantitative detection and clearance validation of residual porcine DNA when manufacturing recombinant therapeutics.
© 2013 Published by Elsevier Ltd on behalf of The International Alliance for Biological Standardization.

1. Introduction

Biopharmaceutical products, such as recombinant protein and viral vaccines, are derived from complex expression systems involving a genetically modified host cell (*i.e.*, bacterial, yeast, or mammalian cell). Among the impurities to be eliminated during downstream purification, residual host-cell DNA and protein are major safety concerns [1,2]. Such impurities must be reduced to an acceptable level in medicinal products that will be administered to patients.

Notably, residual host-cell DNA may be tumorigenic or infectious for a recipient [3,4]. Moreover, risk associated with residual DNA is quantified in terms of a safety factor of a vaccine product [5,6]. Regulatory guidance suggests the risks of oncogenicity and infectivity be mitigated by decreasing both the amount and size of residual DNA [7,8]. The World Health Organization (WHO) and European Union (EU) allow for up to 10 ng/dose of residual DNA. The US Food and Drug Administration (FDA) acceptable residual amount of DNA is 100 pg/dose, as determined by an assay with a detection limit (LOD) of at minimum 10 pg/reaction [9–11]. To confirm the absence of residual host-cell DNA, assessment of residual DNA is one routine item that is monitored prior to release of a final production batch.

Transgenic expression of therapeutic glycoproteins in milk is a novel method for production of biopharmaceutical products [12]. Many therapeutic transgenic proteins (such as C1 Inhibitor, monoclonal antibodies, human alpha-1 antitrypsin and fibrinogen) have been produced in a number of different species (such as goats, rabbits and cows) [13,14]. Recombinant human clotting factor IX (rhFIX) produced from transgenic sow' milk for preclinical animal studies has been established [15]. Skim milk stocks were used for the downstream manufacturing process to purify therapeutic protein. Analysis of residual host-cell DNA has been investigated for recombinant protein expressed by microbial or mammalian cell lines [16–18]; however, no study has examined transgenic animal–derived therapeutic proteins.

The porcine endogenous retrovirus (PERV) in the pig genome is a particular concern, and no definitive data exist for PERV transmission in relation to the clinical use of pig organs [19,20]. A number of PERV integration sites, in the range of 30–50, exist in the genomes of various pig breeds, and at least three PERV subtypes have been documented [21]. For public health issues, a highly sensitive technique is needed to monitor therapeutic products derived from pigs and administered to recipients [22]. To detect

* Corresponding author. Tel.: +886 37 585789; fax: +886 37 585830.
E-mail address: wsr@mail.atit.org.tw (S.-R. Wang).

1045-1056/$36.00 © 2013 Published by Elsevier Ltd on behalf of The International Alliance for Biological Standardization.
http://dx.doi.org/10.1016/j.biologicals.2013.10.005

PERV proviral genes, this study applies a novel quantitative polymerase chain reaction (Q-PCR) technique. This approach will prove useful when assessing residual DNA impurity and when examining host-cell DNA clearance.

2. Materials and methods

2.1. Preparation of reference DNA

The PK15 cells (BCRC 60057, porcine kidney cell) were obtained from the Biosource Collection and Research Center, Taiwan, and maintained in Minimum Essential Medium (MEM) medium with 5% fetal bovine serum, and incubated at 37 °C under 5% CO_2. A commercial kit (Qiagen, Inc., Germany) was used to generate a batch of chromosomal DNA of PK15 cells. The DNA concentration and purity were determined by spectrophotometric analysis at OD260/280.

2.2. Optimization of the quantitative real-time PCR assay

The quantitative real-time polymerase chain reaction (Q-PCR) method was utilized to determine the amount of contaminant DNA in biological material using specific primers and probes for the PERV (GenBank Accession No. AF038601) and porcine beta-actin (GenBank Accession No. AY550069). Sequences of the forward primer and reverse primer of the PERV were 5′-AGC TCC GGG AGG CCT ACT C-3′ and 5′-ACA GCC GTT GGT GTG GTC A-3′, respectively; the probe sequence of the PERV was 5′-FAM-CCA CCG TGC AGG AAA CCT CGA GAC T-TAMRA-3′. Sequences of the forward primer and reverse primer of the beta-actin for porcine were 5′-TGC GGC ATC CAC GAA ACT A-3′ and 5′-GCC GTG ATC TCC TTC TGC AT-3′, respectively; the probe sequence of the beta-actin was 5′-FAM-CTT CAA CTC CAT CAT GAA GTG CGA CGT C-TAMRA-3′. The primers and probes were purified using an Oligonucleotide Purification Cartridge (OPC). The Q-PCR reaction mix contained a final concentration of 300 nM each primer, 200 nM probe, and 1× Taqman gene expression master mix (Applied Biosystems, USA). A water sample was used as the negative control. For the standard curve, serial dilutions of reference DNA were prepared in water and a fixed volume (10 μL) of each dilution was tested as described above. Amplification was conducted in an ABI Prism 7300 Sequence Detection System (Applied Biosystems, USA) with an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C (PERV) or 56 °C (beta-actin).

2.3. Validation study of the quantitative real-time PCR assay

The precision, accuracy, linearity, specificity, detection limit (LOD) and quantitative limit (LOQ) of the assay for quantitative detection of residual porcine DNA were validated according to International Conference on Harmonisation (ICH) guideline for industry [23]. To obtain a standard curve and verify assay sensitivity, serial 10-fold dilutions containing 10^5–0.5 pg PK15 genomic DNA were amplified using the optimized Q-PCR method. A standard curve for quantification was generated by plotting the log of the DNA concentration of reference DNA against the threshold cycle (C_T).

Precision studies to assess repeatability and intermediate precision were conducted to verify that quantification data obtained from multiple tests were uniform. Inter-assay or intra-assay variability was quantified by two independent analyses on different days for standard solutions containing 10^5, 10^3, 10^1 pg of PK15 genomic DNA among three analytical repeats in one run. Accuracy was determined by calculating the DNA recovery percentage between the observed DNA concentration for a sample and the expected DNA concentration of the sample as determined spectrophotometrically, an independent quantification method. The linearity of the analytical procedure was assessed to determine its ability to obtain test results that are directly proportional to the PK15 genomic DNA concentration in a sample. To determine the LOD and LOQ of the analytical procedure, sensitivity studies of the PERV and beta-actin Q-PCR method were performed, respectively. Serial dilutions of 10^5–0.5 pg of PK15 genomic DNA were prepared and amplified using the optimized conditions for generation of a standard curve. Additionally, DNA extracted from rabbit, mouse, cat, hamster, monkey, human cell, yeast, and *Escherichia coli* were used to verify the specificity of PERV primers and probe.

2.4. Data analysis

Following amplification, data were analyzed using the 7300 Real-time System Sequence Detection Software version 1.4 (Applied Biosystems, USA). The standard curve was generated by plotting C_T against the log DNA concentration (pg/reaction) of the DNA standard solutions. The C_T was the cycle at which fluorescence crossed the threshold value. Percentage DNA recovery was calculated using the following equation: (observed DNA concentration − inherent DNA concentration)/DNA spiking concentration × 100%. Tests were deemed acceptable when (i) all three replicate wells of the negative control had undetermined C_T values or C_T values ≥38; (ii) the percentage recovery of quality control solutions were within 30% of their expected values; and (iii) the standard curve had a linear coefficient of determination (R^2) of ≥0.99.

2.5. Residual DNA detection in purified rhFIX derived from transgenic pigs

The rhFIX derived from transgenic pigs was monitored for residual porcine DNA using the PERV Q-PCR method. Residual porcine DNA in samples during different purification process steps was tested as the "un-spiked sample." Samples were spiked with PK15 genomic DNA as "spiked samples" to verify extraction efficiency and the presence of Q-PCR inhibitors. The DNA extracts from the negative control, spiked and un-spiked samples were isolated using the Wako DNA Extractor Kit (Wako, USA). In the Q-PCR process, each sample included a non-template control (NTC), negative control, positive control, spiked and un-spiked samples were used in the nucleic acid amplification reaction conducted in duplicate.

System suitability criteria were applied to the analysis method, including a standard curve coefficient of determination (R^2) of ≥0.99, a slope of the standard curve of −3.1 to −3.7, at least 50–200% recovery of the spiked DNA in the test article, relative standard deviation (RSD) of C_T for replicates of ≤5%, and the C_T of the sentinel negative control and NTC were both undetermined C_T values or were ≥38.

3. Results

3.1. Specificity

The proposed PERV quantitative PCR was applied to genomic DNA from eight species. The DNA extracted from rabbit, mouse, cat, hamster, monkey, human cell, yeast, and bacteria were indistinguishable from the response of the background (NTC) when analyzed with the PERV primer and probe. Porcine DNA, regardless of whether it was from PK15, pig's blood, or sow's milk, generated a dose-dependent response curve (Fig. 1).

3.2. Precision

Intra-assay precision was determined from experimental results of three analyses at different concentration levels (*i.e.*, 10 pg, 10^3 pg,

Download English Version:

https://daneshyari.com/en/article/2034120

Download Persian Version:

https://daneshyari.com/article/2034120

Daneshyari.com